Apolipoprotein C-I binds free fatty acids and reduces their intracellular esterification

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Abstract **Mice that overexpress human apolipoprotein C-I (apoC-I) homozygously (*APOC1*$^{+/+}$ mice) are protected against obesity and show cutaneous abnormalities. Although these effects can result from our previous observation that apoC-I inhibits FFA generation by LPL, we have also found that apoC-I impairs the uptake of a FFA analog in adipose tissue. In this study, we tested the hypothesis that apoC-I interferes with cellular FFA uptake independent of LPL activity. The cutaneous abnormalities of *APOC1*$^{+/+}$ mice were not affected after transplantation to wild-type mice, indicating that locally produced apoC-I prevents lipid entry into the skin. Subsequent in vitro studies with apoC-I-deficient versus wild-type macrophages revealed that apoC-I reduced the cell association and subsequent esterification of [^{3}H]oleic acid by ~35% ($P < 0.05$). We speculated that apoC-I binds FFA extracellularly, thereby preventing cell association of FFA. We showed that apoC-I was indeed able to mediate the binding of oleic acid to otherwise protein-free VLDL-like emulsion particles involving electrostatic interaction. We conclude that apoC-I binds FFA in the circulation, thereby reducing the availability of FFA for uptake by cells. This mechanism can serve as an additional mechanism behind the resistance to obesity and the cutaneous abnormalities of *APOC1*$^{+/+}$ mice.**—Westerterp, M., J. F. P. Berbée, D. J. M. Delsing, M. C. Jong, M. J. J. Gijbels, V. E. H. Dahlmans, E. H. Offerman, J. A. Romijn, L. M. Hayekes, and P. C. N. Rensen. **Apolipoprotein C-I binds free fatty acids and reduces their intracellular esterification.** ***J. Lipid Res.*** **2007.** 48: **1353–1361.**

Supplementary key words lipoprotein • macrophage • skin • lipoprotein lipase • triglyceride

Apolipoprotein C-I (apoC-I) is a 6.6 kDa apolipoprotein that is synthesized mainly in the liver but also in the skin, adipose tissue, central nervous system, kidney, and spleen (1). It circulates in plasma at a concentration of ~6 mg/dl and is present mainly on chylomicrons, VLDLs, and HDLs (2). To study the role of apoC-I in lipoprotein metabolism, we have generated apoC-I-deficient (*apoc1*$^{-/-}$) (3), *apoe*$^{-/-}$*apoc1*$^{-/-}$ (4), human transgenic hemizygous apoC-I (*APOC1*$^{+/0}$), homozygous apoC-I (*APOC1*$^{+/+}$) (5), and *apoe*$^{-/-}$*APOC1*$^{+/0}$ mice (6). Endogenous apoC-I expression in wild-type and *apoe*$^{-/-}$ mice and overexpression of human apoC-I in wild-type and *apoe*$^{-/-}$ mice have consistently led to increased triglyceride (TG) levels, mainly caused by the apoC-I-induced inhibition of LPL (6–8). In addition, *APOC1*$^{+/+}$ mice exhibit reduced adipose tissue stores and are protected against obesity as induced by diet and on an *ob/ob* background (9). Furthermore, *APOC1*$^{+/+}$ mice lack subdermal fat, show decreased levels of wax esters and TG in their skin and cutaneous abnormalities, including epidermal hyperplasia/hyperkeratosis, and atrophic sebaceous and meibomian glands. These abnormalities are reflected by a dry skin, crusts around the eyes, hair loss at a young age, and lack of sebum and meibum production (10). Sebum is suggested to prevent water loss from the skin (10), whereas meibum prevents evaporation of the aqueous component of the tear (11), both by formation of a lipid layer on the fluid in the skin or of the tear.

Reduced systemic LPL activity in *APOC1*$^{+/+}$ mice contributes to diminished availability of FFA for esterification into TG for storage in adipose tissue and into TG and wax

Abbreviations: *acat1*$^{-/-}$, acyl-CoA:cholesterol acyltransferase 1-deficient; apoC-I, apolipoprotein C-I; *APOC1*$^{+/+}$, homozygous human apoC-I transgenic; *APOC1*$^{+/0}$, hemizygous human apoC-I transgenic; *apoc1*$^{-/-}$, apoC-I-deficient; BMIPP, 15-(*p*-iodophenyl)-3-(*R,S*)-methylpentadecanoic acid; CE, cholesteryl ester; *dgat1*$^{-/-}$, acyl-CoA:diacylglycerol acyltransferase 1-deficient; FAR, fatty acyl-CoA reductase; FPLC, fast-performance liquid chromatography; GAG, glycosaminoglycan; *lpl*$^{-/-}$, LPL-deficient; LPS, lipopolysaccharide; *scd1*$^{-/-}$, stearoyl-CoA desaturase 1-deficient; TG, triglyceride.

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Manuscript received 17 January 2007 and in revised form 2 March 2007.
Published, JLR Papers in Press, March 5, 2007.
DOI 10.1194/jlr.M700024-JLR200

esters that are major components of sebum in the skin (10). However, it is likely that apoC-I also interferes with FFA availability by direct binding to FFA. *APOC1*$^{+/+}$ mice have 4-fold increased levels of FFA in plasma compared with their wild-type littermates (10), and the uptake of the FFA analog 15-(*p*-iodophenyl)-3-(*R,S*)-methylpentadecanoic acid (BMIPP) by adipose tissue is reduced in *APOC1*$^{+/+}$*ob/ob* mice compared with their *ob/ob* littermates (9). The hypothesis that apoC-I directly interacts with FFA is further supported by our recent finding that apoC-I binds to lipopolysaccharide (LPS), mainly as the consequence of the positively charged lysine (K) residues in the LPS-binding motif KVKEKLK of apoC-I, which presumably interact with the negatively charged lipid A moiety of LPS (12). As FFAs are also negatively charged at physiological pH, the binding properties of apoC-I to FFAs might parallel those to LPS.

Therefore, the aim of this study thus was to evaluate whether locally produced apoC-I reduces FFA esterification in cells by reducing FFA availability as a consequence of extracellular association with FFA. We showed first by skin transplantation from *APOC1*$^{+/+}$ mice to wild-type mice that the effects of apoC-I on FFA esterification in the skin were local, as the skin phenotype did not change upon relief of systemic LPL inhibition. Then, we demonstrated that apoC-I reduced the esterification of FFA into TG and cholesteryl esters (CEs) in macrophages, cells that naturally accumulate lipids, as related to direct extracellular binding of apoC-I to FFA. The effects of apoC-I on FFA binding and intracellular esterification were dependent on the positively charged lysine residues in the KVKEKLK motif of apoC-I. From these data, we conclude that apoC-I binds to FFA as a consequence of its lysine-rich sequence, which results in reduced influx of FFA into cells by direct inhibition of cellular FFA influx.

MATERIALS AND METHODS

Animals

Apoc1$^{-/-}$ (3), *APOC1*$^{+/0}$, and *APOC1*$^{+/+}$ (line 11/1) mice (5, 10) were generated as described previously and were backcrossed at least eight times to the C57Bl/6 background. Skin transplantation experiments were performed in 12–13 week old wild-type and *APOC1*$^{+/+}$ male mice. Peritoneal macrophages were isolated from 12 week old *apoc1*$^{-/-}$ and wild-type (*apoc1*$^{+/+}$) male mice. Blood for FFA and apoC-I measurement was drawn from 10 week old wild-type, *APOC1*$^{+/0}$, and *APOC1*$^{+/+}$ female mice. Animal experiments were performed at the Gaubius Laboratories of the Netherlands Organization for Applied Scientific Research-Quality of Life. All experimental protocols were approved by the institutional Ethics Committee for Animal Experiments.

Skin transplantation

Donor mice were euthanized, dorsal skin (dermis and epidermis) was removed, and excess fat and blood vessels were stripped off. The recipient mice were anesthetized with Hypnorm (1:20, 120 μl/mouse; Janssen Pharmaceutica, Tilburg, The Netherlands). Two circular pieces of dorsal skin, ~1 cm in diameter, were aseptically removed down to the pannus carnosus. The donor skin was cut to fit the transplant bed and held in place using six sutures. The transplant was covered with Op-Site (Smith and Nephew, Hull, England) and protected by gauze held in place with 3M surgical foam tape. The tape was kept on for at least 2 weeks. *APOC1*$^{+/+}$ mice were transplanted with skin grafts from wild-type mice, and inversely, wild-type mice were transplanted with skin grafts from *APOC1*$^{+/+}$ mice. As a control, wild-type mice were transplanted with skin grafts from wild-type mice. Six weeks after transplantation, mice were euthanized, and pieces of skin tissue were fixed in 10% neutral-buffered formalin. Subsequently, skin tissue was embedded in paraffin, and 3 μm sections were made and routinely stained with hematoxylin-phloxine-saffron.

ApoC-I mutant synthesis

An apoC-I mutant in which the lysines of the C-terminal KVKEKLK sequence, involved in the LPS binding properties of apoC-I, were replaced by alanines (A) (giving AVAEALA) was synthesized by the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center (Dallas, TX) (purity ⩾ 95%) (12).

Macrophage studies

Apoc1$^{-/-}$ and wild-type mice were injected intraperitoneally with 1 ml of 3% Brewer's thioglycollate medium (Difco) to induce the infiltration of macrophages. After 4 days, macrophages were isolated, plated, and cultured for 24 h. *Apoc1*$^{-/-}$ and wild-type macrophages were loaded with 50 μg/ml acetylated LDL for 48 h to enlarge the intracellular lipid pool. Subsequently, cells were washed and incubated with 0.1 M oleic acid (Sigma, St. Louis, MO), 2 μCi/ml [^{3}H]oleic acid (Amersham Biosciences, Little Chalfont, UK), and 0.1% fatty acid-free BSA (Sigma) for 0, 15, 30, 45, 75, and 120 min. Alternatively, wild-type macrophages were incubated for 45 min with and without 5 mM apoC-I or apoC-I mutant, 5 mM oleic acid, 0.1 μCi/ml [^{3}H]oleic acid, and 0.05% fatty acid-free BSA.

Then, all cells were lysed in water, and the cellular protein content (13) as well as cellular ^{3}H activity were measured, with the ratio ^{3}H activity/cell protein indicating the association of [^{3}H]oleic acid with the cells. To determine the extent of [^{3}H]oleic acid esterification into TG and CE in *apoc1*$^{-/-}$ and wild-type macrophages, cellular lipids were extracted according to Bligh and Dyer (14) and separated by high-performance thin-layer chromatography. After staining with color reagent [5 g of $MnCl_2 \cdot 4H_2O$, 32 ml of 95–97% H_2SO_4 added to 960 ml of CH_3OH/H_2O (1:1, v/v)], the bands corresponding to CE and TG were scraped off the plates, counted for ^{3}H activity, and corrected for cellular protein content.

Analysis of mouse plasma

Blood from wild-type, *APOC1*$^{+/0}$, and *APOC1*$^{+/+}$ mice was collected by tail bleeding into chilled paraoxon (Sigma)-coated capillary tubes to prevent ongoing in vitro lipolysis. The tubes were placed on ice and centrifuged at 4°C, and the obtained plasma was snap-frozen in liquid nitrogen and stored at −20°C. To determine the association of apoC-I and FFA with VLDL, plasma was injected onto a Superose 6 HR 10/30 column, and the obtained fractions were assayed for FFA using the NEFA-C kit (Wako Chemicals, Neuss, Germany) and for apoC-I using the apoC-I ELISA as described below.

Preparation of TG-rich VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared and characterized as described previously (15, 16). In short, 100 mg of lipid at a weight ratio of egg yolk phosphatidylcholine-triolein-lysophosphatidylcholine-cholesteryl oleate-cholesterol of 22.7:70:2.3:3.0:2.0 was sonicated at 10 μm output using a Soniprep 150

(MSE Scientific Instruments, Crawley, UK). An emulsion fraction containing 80 nm emulsion particles was obtained by consecutive density gradient ultracentrifugation steps and used for subsequent experiments. The TG content of the emulsions was determined using the commercially available enzymatic kit 1488872 (Roche Molecular Biochemicals, Indianapolis, IN). Emulsions were stored at 4°C under argon and were used within 14 days.

ApoC-I FFA binding experiments

ApoC-I (5, 10, 20, and 40 mg/dl) or the apoC-I mutant (9.7 mg/dl; molar concentration equal to 10 mg/dl apoC-I) was associated with emulsion particles (6 mM TG) by incubation in PBS (pH 7.4) for 30 min at 37°C. Subsequently, 2 mM oleic acid complexed with 5 mg/ml fatty acid-free BSA in PBS (pH 7.4) was added (total volume, 80 μl) and was allowed to associate with the (apoC-I-laden) emulsion particles for an additional 30 min at 37°C. To determine the association of oleic acid with the apoC-I-laden emulsion particles, the mixture was injected onto a Superose 6 HR 10/30 column (Akta System; Amersham Pharmacia Biotech, Piscataway, NJ) and eluted at a constant flow rate of 50 μl/min PBS, 1 mM EDTA (Sigma), pH 7.4. During fast-performance liquid chromatography (FPLC), fractions of 50 μl were collected and assayed for apoC-I using the apoC-I ELISA and for FFA as described above.

ApoC-I ELISA

The apoC-I concentration in FPLC fractions and in plasma from *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice was quantified using a human apoC-I sandwich ELISA as described previously (6). In short, a polyclonal goat anti-human apoC-I antibody (Academy Biomedical Co., Houston, TX) was coated overnight onto Costar medium binding plates (Corning, Inc., New York, NY) (dilution, 1:10,000) at 4°C and incubated with the diluted pool of FPLC fractions containing the emulsion particles (dilution, 1:10,000) or mouse plasma (dilution, 1:16,000) for 2 h at 4°C. Subsequently, HRP-conjugated polyclonal goat anti-human apoC-I antibody (dilution, 1:500; Academy Biomedical Co.) was added and incubated for 2 h at room temperature. After extensive washing, HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 20 min at room temperature. Human apoC-I (Labconsult, Brussels, Belgium) was used as a standard.

Statistics

All data are presented as means ± SD. The general linear model was used to compare the trends between the [^{3}H]oleate ester generation curves of *apoc1*$^{+/+}$ (wild-type) and *apoc1*$^{-/-}$ macrophages. The Mann-Whitney nonparametric test for two independent samples was used to define differences between data sets from experimental groups. The criterion for significance was set at $P < 0.05$. Statistical analyses were performed using SPSS 11.5 (SPSS, Inc., Chicago, IL).

RESULTS

Effect of transplantation on the phenotype of the *APOC1*$^{+/+}$ skin

To investigate whether the cutaneous abnormalities of the *APOC1*$^{+/+}$ mice were the consequence of either systemic or local apoC-I expression, we transplanted skin grafts from *APOC1*$^{+/+}$ mice into wild-type mice and from wild-type mice into *APOC1*$^{+/+}$ mice. After 6 weeks, we euthanized the mice to examine the histology of the skin with respect to the sebaceous glands. Normal sebaceous glands contain a light core indicating the presence of fat, which is absent in atrophic sebaceous glands. The cutaneous abnormalities including the atrophic sebaceous glands of *APOC1*$^{+/+}$ mice were not alleviated upon transplantation to normolipidemic wild-type mice (**Fig. 1A**, D). Inversely, the normal phenotype of the skin of wild-type mice, containing normal sebaceous glands, was not affected upon transplantation to hyperlipidemic *APOC1*$^{+/+}$ mice (Fig. 1B, E). As a control, wild-type skin was transplanted into wild-type mice that did not show any effect on the appearance of the skin and the sebaceous glands (Fig. 1C, F). The effect of apoC-I on the phenotype of the skin was thus dependent on the local synthesis and concentration of apoC-I. Collectively, these data suggest that the effect of apoC-I on the skin phenotype in *APOC1*$^{+/+}$ mice is a local cellular effect, rather than a result of systemic LPL inhibition.

Effect of apoC-I on the uptake and esterification of [^{3}H]oleic acid in macrophages

Because the skin of *APOC1*$^{+/+}$ mice does not produce sebum (10), and TG and wax esters, major components of sebum, are formed as a result of FFA esterification (10), we investigated whether the effects of apoC-I on the appearance of the skin might be the result of a reduced esterification of FFA. Hereto, we used macrophages, cells that naturally accumulate lipids and also infiltrate into the inflamed skin of *APOC1*$^{+/+}$ mice (10) and into adipose tissue (17).

First, we examined the effect of endogenous apoC-I on the esterification of oleic acid into CE and TG using *apoc1*$^{-/-}$ and *apoc1*$^{+/+}$ (wild-type) macrophages. Although the association of [^{3}H]oleic acid with cells of both genotypes increased time-dependently, the cell association was consistently ~25% lower in *apoc1*$^{+/+}$ versus *apoc1*$^{-/-}$ macrophages ($P < 0.05$) (**Fig. 2**). After separation of intracellular lipids, it appeared that this decrease was reflected by a decreased esterification of [^{3}H]oleic acid into both TG (**Fig. 3A**) and CE (Fig. 3B). After 120 min of incubation, apoC-I decreased the esterification of [^{3}H]oleic acid into TG by 36% ($P < 0.05$) (Fig. 3A) and the esterification of [^{3}H]oleic acid into CE by 35% (Fig. 3B). Furthermore, the trends of the curves for the esterification of [^{3}H]oleic acid into TG and CE were both significantly different between *apoc1*$^{-/-}$ and *apoc1*$^{+/+}$ macrophages ($P < 0.05$), indicating that apoC-I expression reduced the overall rate of oleic acid esterification (Fig. 3A, B).

Because peritoneal macrophages from *APOC1*$^{+/+}$ mice showed a very low level of secretion of human apoC-I in the medium (15–20 ng/ml under the same experimental conditions), apoC-I overexpression appeared not to be a relevant strategy to study the effect of human apoC-I on FFA esterification. Because the apoC-I propeptide contains a signal peptide, newly synthesized apoC-I is secreted from cells (18). Therefore, we used the addition of extracellular apoC-I as an approach to study the effect of human apoC-I on FFA esterification in macrophages. Addition of apoC-I reduced the association of [^{3}H]oleic acid with the cells by 32% ($P < 0.05$) (**Fig. 4**). In contrast, the apoC-I mutant was unable to reduce the cell association of

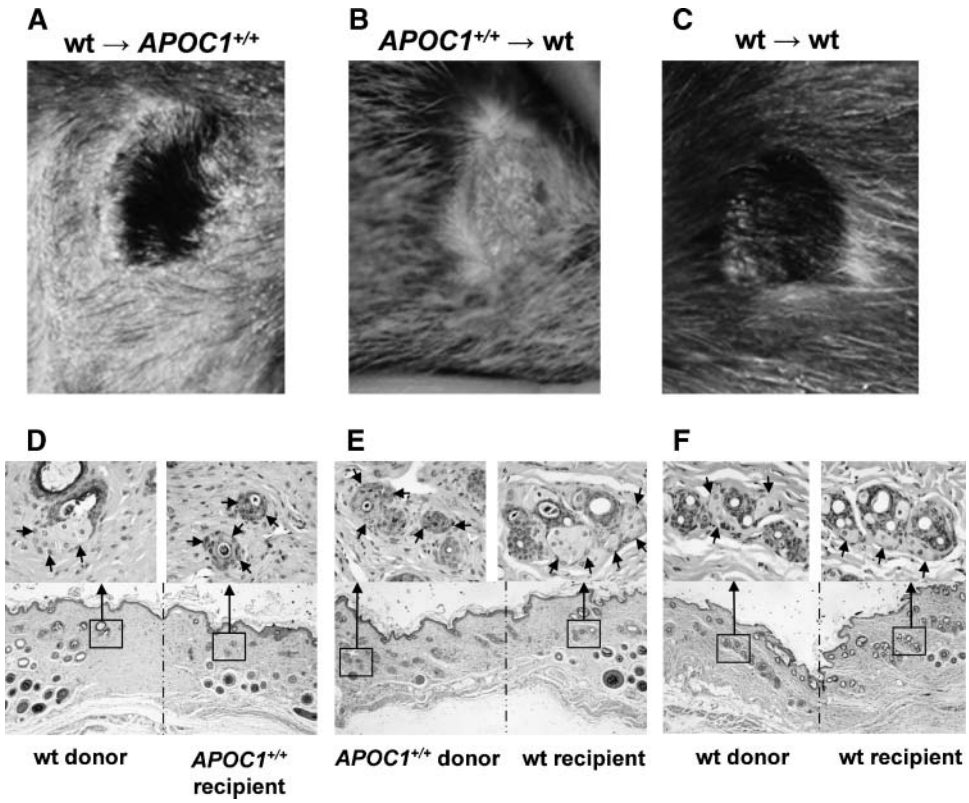

Fig. 1. Effect of transplantation on the phenotype of homozygous human apolipoprotein C-I transgenic (*APOC1*$^{+/+}$) skin. *APOC1*$^{+/+}$ mice were transplanted with skin from wild-type (wt) mice (A, D), wild-type mice were transplanted with skin from *APOC1*$^{+/+}$ mice (B, E), and wild-type mice were transplanted with skin from wild-type mice (C, F). The skin of the mice is shown at six weeks after plantation (A–C). Mice were euthanized, and the skin was examined histologically (D–F). Bottom pictures indicate an overview (40 × magnification), and pictures of higher magnification (400 ×) are presented on top. Note the atrophic and normal sebaceous glands of the *APOC1*$^{+/+}$ and the wt skin, respectively (arrowheads).

[^{3}H]oleic acid, indicating that the positively charged lysine residues in the KVKEKLK motif of apoC-I play a major role in the inhibiting the effect of apoC-I on the association of [^{3}H]oleic acid with macrophages.

Effect of apoC-I on FFA association with VLDL-like emulsion particles

Because *1*) the esterification of oleic acid both into TG and CE was decreased upon expression of apoC-I, *2*) apoC-I is secreted mainly from cells (18), and *3*) the extracellular addition of apoC-I reduced the association of oleic acid with macrophages, we hypothesized that apoC-I could interact with oleic acid, thereby preventing its uptake by macrophages for subsequent esterification. Such an interaction between apoC-I and FFA could explain the increased FFA levels in *APOC1*$^{+/+}$ and *APOC1*$^{+/0}$ mice compared with wild-type mice (6, 10).

We first determined the distribution of FFA and apoC-I over lipoproteins in plasma of wild-type, *APOC1*$^{+/0}$, and *APOC1*$^{+/+}$ mice. In wild-type mice, the majority of FFA is bound to serum albumin and only a minor fraction associates with VLDL (<5%). In *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice, serum albumin-associated FFA levels increased, indicating increased FFA saturation of serum albumin. In addition, the association of FFA with VLDL/IDL was highly increased in *APOC1*$^{+/0}$ mice (~25%) and *APOC1*$^{+/+}$ mice (~62%), corresponding with increased apoC-I levels on VLDL/IDL (7.9 and 59.1 mg/dl in *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice, respectively) (**Fig. 5**). Thus, these results suggest that apoC-I bridges FFA to VLDL/IDL particles.

To investigate whether apoC-I directly mediates the association of FFA with VLDL, we determined the effect of apoC-I and the apoC-I mutant, bound to otherwise protein-free VLDL-like emulsion particles, on the association of oleic acid with the emulsion particles after incubation with BSA saturated with oleic acid. After incubation, the emulsion was separated from BSA by FPLC and the fractions were assayed for apoC-I and FFA. The doses of apoC-I or the apoC-I mutant added to the emulsion were almost completely recovered on the emulsion (>95%) after separation of the mixture over the Superose 6 column, as measured using the apoC-I ELISA (results not shown). Although the emulsion itself was able to bind oleic acid, addition of apoC-I (10 mg/dl) increased oleic acid association by 90% ($P < 0.05$) (**Fig. 6A**). In contrast, an equimolar concentration of the apoC-I mutant was ineffective. In fact, the effect of apoC-I on oleic acid binding to the emulsion particles was dose-dependent (Fig. 6B). These data indicate that apoC-I can indeed enhance the binding of FFA to VLDL.

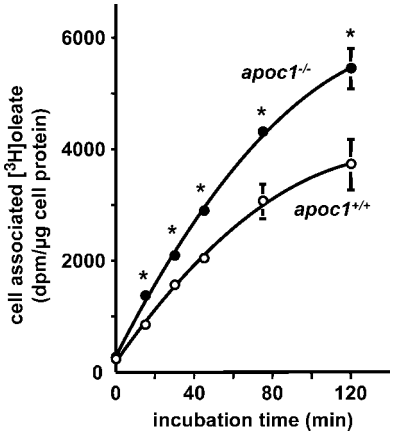

Fig. 2. Effect of endogenous apolipoprotein C-I (apoC-I) expression on the association of [^{3}H]oleic acid with macrophages. Peritoneal apoC-I-deficient (*apoc1*$^{-/-}$; black symbols) and *apoc1*$^{+/+}$ (white symbols) macrophages were isolated and cultured. After 24 h, medium was withdrawn and macrophages were loaded with 50 µg/ml acetylated LDL for 48 h. Subsequently, cells were washed and incubated with 0.1 mM oleic acid and 2 µCi/ml [^{3}H]oleic acid in the presence of 0.1% fatty acid-free BSA. At the indicated times, cells were washed and lysed. In the cell lysates, protein and ^{3}H activity were determined. Values are presented as means ± SD (n = 3). * $P < 0.05$.

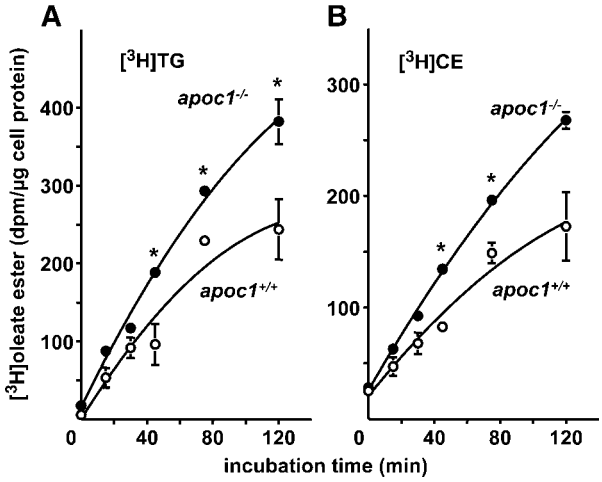

Fig. 3. Effect of endogenous apoC-I expression on the esterification of [^{3}H]oleic acid into triglycerides (TGs) and cholesteryl esters (CEs). Peritoneal *apoc1*$^{-/-}$ (black symbols) and *apoc1*$^{+/+}$ (white symbols) macrophages were isolated and cultured. After 24 h, medium was withdrawn and macrophages were loaded with 50 µg/ml acetylated LDL for 48 h. Subsequently, cells were washed and incubated with 0.1 mM oleic acid and 2 µCi/ml [^{3}H]oleic acid in the presence of 0.1% fatty acid-free BSA. At the indicated times, cells were washed and lysed and the protein content of the cell lysate was determined. Subsequently, lipids were extracted from cell lysates and separated by high-performance thin-layer chromatography. Bands for TG (A) and CE (B) were scraped off and counted for ^{3}H activity. Values are presented as means ± SD (n = 3). * $P < 0.05$.

DISCUSSION

We showed previously that *APOC1*$^{+/+}$ mice are hyperlipidemic, resistant to diet-induced obesity, and show cutaneous abnormalities (9, 10). These effects can be explained by LPL inhibition, resulting in the generation of less FFA for subsequent uptake by adipose tissue and skin. In this study, we present an additional mechanism: by the direct association of FFA to apoC-I in plasma, apoC-I can inhibit the uptake and subsequent intracellular esterification of FFA. We show that these effects of apoC-I are dependent on positively charged lysine residues within the previously identified LPS binding element of apoC-I.

The explanation that LPL inhibition may underlie the phenotype of the *APOC1*$^{+/+}$ mouse regarding cutaneous abnormalities and protection against obesity is supported by the findings that adipose tissue-specific LPL-deficient (*lpl*$^{-/-}$) mice are protected against obesity on the *ob/ob* background and that whole body *lpl*$^{-/-}$ pups lack, in their short life span of only 18 h, subcutaneous fat (19, 20). Thus, reduced LPL activity accounts for reduced adipose tissue stores. Although no effects on the skin of *lpl*$^{-/-}$ mice can be studied as a result of the short life span of *lpl*$^{-/-}$ pups (19), LPL is expressed in the dermal layer (21) and may affect FFA uptake in the skin. Thus, reduced LPL activity may, at least partially, explain the reduced obesity, the lack of subcutaneous fat, and the cutaneous abnormalities in *APOC1*$^{+/+}$ mice.

Several lines of evidence indicate that an additional mechanism can explain the reduced FFA uptake by adipose tissue and skin. For example, the uptake of the FFA analog BMIPP by adipose tissue was reduced by human apoC-I expression on the *ob/ob* background (9) and plasma FFA levels are increased in *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice compared with wild-type mice (6, 10). As the apoC-I propeptide contains a signal peptide, resulting in the secretion of newly synthesized apoC-I from cells

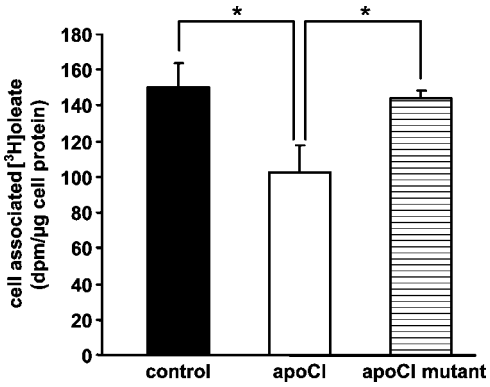

Fig. 4. Effect of exogenous apoC-I on the association of [^{3}H]oleic acid with macrophages. Peritoneal wild-type macrophages were isolated and cultured. After 24 h, medium was withdrawn and macrophages were loaded with 50 µg/ml acetylated LDL for 48 h. Subsequently, cells were washed and incubated without (black bar) or with 5 mM apoC-I (white bar) or 5 mM apoC-I mutant (striped bar) in the presence of 5 mM oleic acid, 0.1 µCi/ml [^{3}H]oleic acid, and 0.05% fatty acid-free BSA for 45 min. Subsequently, cells were washed and lysed. Cell protein and ^{3}H activity of the cell lysate were determined. Values are presented as means ± SD (n = 3). * $P < 0.05$.

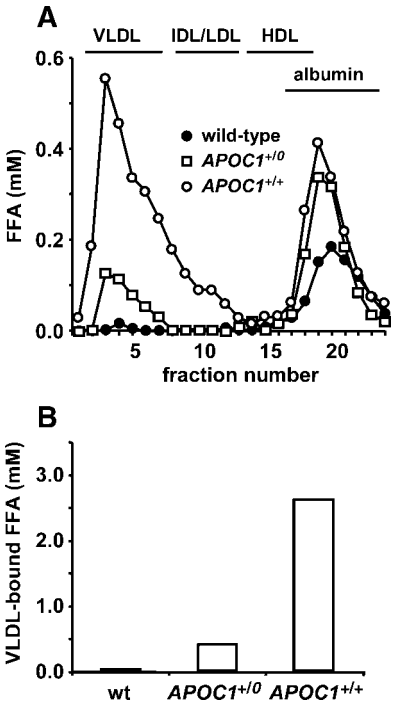

Fig. 5. Effect of apoC-I on FFA association with VLDL in plasma. Plasma from 4 h fasted wild-type (wt; black circles), hemizygous human apoC-I transgenic (*APOC1*$^{+/0}$; white squares), and *APOC1*$^{+/+}$ (white circles) mice was pooled per genotype. Lipoproteins were size-fractionated by fast-performance liquid chromatography (FPLC) on a Superose 6 column, and the individual fractions were assayed for FFA (A).The association of FFA with VLDL/IDL was then calculated (B). IDL, intermediate density lipoprotein.

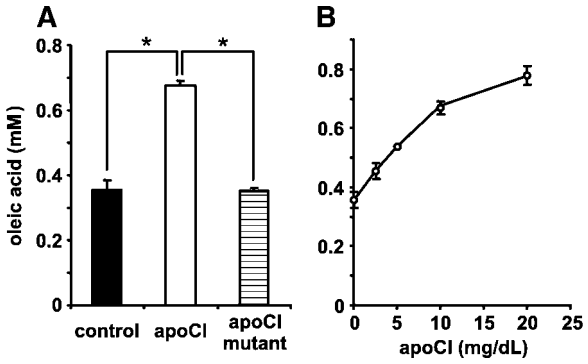

Fig. 6. Effect of apoC-I on oleic acid association with VLDL-like emulsion particles. The VLDL-like TG emulsion (6 mM) was incubated without (black bar) and with 10 mg/dl apoC-I (white bar) or 9.7 mg/dl apoC-I mutant (equimolar concentration to apoC-I) (striped bar) for 30 min at 37°C (A) or with the indicated concentrations of apoC-I (B). Subsequently, 2 mM oleic acid complexed with 5 mg/ml fatty acid-free BSA was added. After another 30 min, the oleic acid bound to the emulsion was separated from the oleic acid bound to BSA using FPLC. In the pooled fractions, the association of oleic acid with the emulsion was calculated. Values are presented as means ± SD (n = 3). * $P < 0.05$.

(18), apoC-I may interact with FFA extracellularly. Furthermore, apoC-I binds to the LPS, presumably as a consequence of an electrostatic interaction between the negatively charged phosphate groups in the lipid A moiety of LPS and the four positively charged lysine residues in the LPS binding motif KVKEKLK of apoC-I (12). Similarly, FFAs contain negatively charged carboxyl groups at physiological pH, suggesting that apoC-I might bind to FFAs in a similar manner. Therefore, we hypothesized that apoC-I prevents the influx of FFAs into cells as a result of binding to FFAs in plasma.

We first investigated whether the cutaneous abnormalities of *APOC1*$^{+/+}$ mice are caused by local apoC-I production by performing skin transplantation studies. These studies showed no effects on the phenotype of the skin grafts, indicating that apoC-I reduced lipid entry into the skin and caused the cutaneous abnormalities of the *APOC1*$^{+/+}$ mice via local effects, rather than via inhibition of systemic LPL activity. We then investigated whether apoC-I, either synthesized endogenously or added exogenously, can affect the esterification of extracellularly added oleic acid in cells. We used macrophages that naturally accumulate lipids and can infiltrate both the skin (10) and adipose tissue (17). It appeared that both endogenous and exogenous apoC-I reduced the association of [^{3}H]oleic acid with macrophages, which was reflected by a decreased esterification of [^{3}H]oleic acid into TG and CE. The finding that the incorporation of [^{3}H]oleic acid into TG was greater than into CE can be explained by the presence of three esterified fatty acid chains in TG versus one in CE. These effects of apoC-I were dependent on the positively charged lysine residues in the LPS binding element KVKEKLK of apoC-I, as the apoC-I mutant, containing neutral alanine residues instead (AVAEALA), was ineffective.

Based on these findings, we speculated that the apoC-I-mediated reduction of FFA influx into cells would lead to decreased intracellular FFA levels and increased extracellular FFA levels in plasma. To gain more insight into the validity of such a mechanism, we investigated whether the presence of apoC-I on particular lipoproteins would cause this effect, as apoC-I in plasma is mainly bound to lipoproteins (2). In plasma from *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice, a large fraction of FFA appeared to be associated with VLDL (25% and 62%, respectively), suggesting that apoC-I is able to bridge FFA to VLDL. Subsequent in vitro experiments indeed showed that apoC-I, associated with VLDL-like TG-rich emulsion particles, bound to oleic acid dose-dependently, also dependent on the positively charged lysine residues in the LPS binding element KVKEKLK of apoC-I.

Collectively, these data indicated that apoC-I on VLDL binds to FFA in the circulation, thereby preventing the uptake and subsequent esterification of FFA by cells, which is dependent on the lysine residues in the LPS binding element KVKEKLK of apoC-I. The positive lysine residues contribute to the high isoelectric point of apoC-I (6.5) (22), which decreases to 4.8 upon replacement by neutral alanine residues (12). In fact, the isoelectric point of apoC-I is the highest of all known apolipoproteins (22); therefore, we postulate that this property to bind to FFA is

specific for apoC-I and completely dependent on the electrostatic interaction between apoC-I and FFA.

We previously found that apoC-I can directly inhibit LPL activity (6). Our present finding that apoC-I binds to FFA suggests that apoC-I may also interfere with LPL activity via product inhibition. Indeed, in vitro studies have demonstrated that FFAs abolish the LPL-stimulating effects of apoC-II (23) and impede binding of LPL to its substrate, lipid droplets (24, 25). In addition, FFAs have been demonstrated to displace LPL from cultured endothelial cells in vitro (26) and have been suggested to dissociate LPL from its binding to endothelial proteoglycans if FFAs are more rapidly generated than used by the local tissue (27). In fact, the positively charged amino acid residues arginine (R) and lysine (28, 29) within the RLTRKRGLK motif of apoB appeared to be required for the association of VLDL/LDL with the cell surface glycosaminoglycans (GAGs) on the endothelium, where LPL is also located (30, 31). Conversely, negatively charged FFAs have been demonstrated to disrupt the binding of apoE-supplemented TG emulsions bound to heparin-agarose, as a model for cell surface heparan GAG, in vitro (32). Because binding of apoC-I to FFA depends on the presence of the positively charged lysine residues within the KVKEKLK motif of apoC-I, the electrostatic interaction of negatively charged FFAs associated with these positively charged lysine residues may shield the positively charged apoC-I, thereby decreasing its affinity for the GAG. This might also explain the apoC-I-induced LPL inhibition in *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice.

In addition to *APOC1*$^{+/+}$ mice, stearoyl-CoA desaturase 1-deficient (*scd1*$^{-/-}$) and acyl CoA:diacylglycerol acyltransferase 1-deficient (*dgat1*$^{-/-}$) mice show cutaneous abnormalities and atrophic sebaceous glands, lack sebum and meibum production, and are protected against obesity on the *ob/ob* background (*scd1*$^{-/-}$), in diet-induced obesity (*dgat1*$^{-/-}$), or when bred on the Agouti yellow background (*dgat1*$^{-/-}$) (33–37). Furthermore, acyl-CoA:cholesterol acyltransferase 1-deficient (*acat1*$^{-/-}$) mice show decreased sebum production and lack meibum production (11). The mechanisms underlying these findings in the various mouse models may share similarities. ApoC-I binds to FFAs in plasma, thereby preventing the cellular uptake of FFAs (this study), and SCD1 is the rate-limiting enzyme in the biosynthesis of MUFAs (35). Overexpression of apoC-I and deficiency of SCD1 thus both result in reduced levels of intracellular FAs. DGAT1 and ACAT1 catalyze the rate-limiting steps of TG and CE synthesis, respectively, exhibit wax synthase activity (38, 39), and are downregulated when intracellular FFAs are scarce (40, 41). We thus postulate that apoC-I reduces the availability of FFA substrate for esterification into TG by DGAT1 in adipose tissue, contributing to decreased development of obesity, and into TG by DGAT1 and CE by ACAT1 in macrophages, as observed in this study. Furthermore, apoC-I reduces the esterification of FFAs into wax esters by DGAT1 and ACAT1 in the skin, contributing to decreased sebum and meibum production. In fact, FFAs are first converted to fatty alcohols catalyzed by fatty acyl-CoA reductase 1 and 2 (FAR1 and FAR2) in the sebaceous or meibomian gland and subsequently esterified into wax esters catalyzed by wax synthase enzymes, also including acyl-CoA:monoacylglycerol acyltransferase 1 and 2, DGAT2, and ACAT2 (39, 42). ApoC-I might thus also reduce the activity of these enzymes; however, it is unlikely that apoC-I interacts with SCD1, FAR1 or FAR2, or enzymes that exhibit wax synthase activity, because these enzymes are integral intracellular membrane proteins (35, 39–43) and apoC-I is secreted from cells (18).

It has been speculated that lack of sebum and meibum production in *APOC1*$^{+/+}$ mice is attributable to increased cholesterol levels in their skin, which might lead to cytotoxicity resulting in atrophy of sebaceous and meibomian glands (11). However, hemizygous *APOC1*$^{+/0}$ mice, expressing similarly increased levels of cholesterol in the epidermis compared with their homozygous *APOC1*$^{+/+}$ littermates, show less reduced levels of wax diesters and TG (10). *APOC1*$^{+/0}$ mice did not show sebaceous gland atrophy but only reduced sebum production, with an appearance of the skin similar to that of wild-type mice (10). These results indicate that the lack of sebum production cannot solely be the consequence of high cholesterol levels in the epidermis, at least not upon apoC-I overexpression.

Because *APOC1*$^{+/+}$ mice, but not *scd1*$^{-/-}$, *dgat1*$^{-/-}$, and *acat1*$^{-/-}$ mice (11, 33, 35), exhibit skin lesions containing extensive infiltration of macrophages into the epidermis (10), additional factors may play a role in the cutaneous abnormalities of *APOC1*$^{+/+}$ mice. We recently showed that apoC-I augments the inflammatory response induced by LPS injections or inoculation with *Klebsiella pneumoniae* (12). As sebum is suggested to prevent bacteria from persisting in the hair canal, and *APOC1*$^{+/+}$ mice lack sebum production (10), apoC-I may augment the inflammatory response to those bacteria, resulting in the influx and activation of macrophages and, thus, the inflammatory phenotype of the skin. In addition, extracellular FFAs associated with apoC-I may bind to the Toll-like receptor 4 and thereby induce the activation of the nuclear factor κB pathway (43, 44).

Thus, the expression level of human apoC-I in mice correlates positively with plasma FFA levels, and human apoC-I expression reduces adiposity and the production of sebum and meibum. Meibomian gland dysfunction in mice is a condition similar to dry eye syndrome in humans (11), and meibomian and sebaceous glands are commonly affected together in humans. Meibomian keratoconjunctivitis is frequently associated with sebaceous gland diseases such as seborrhoea sicca (45). Thus, it would be interesting to determine whether apoC-I plays a causal role in these diseases and also in obesity in humans.

Collectively, we conclude that binding of apoC-I to FFA accounts for the reduced uptake and subsequent esterification of FFA in cells. In addition, the physical association between apoC-I and FFA may result in a reduction of LPL activity by product inhibition, further reducing the availability of FFA. As a consequence, meibum and sebum production and the development of obesity are reduced in mice that overexpress human apoC-I.

This work was performed in the framework of the Leiden Center for Cardiovascular Research and supported by the Netherlands Organization for Scientific Research (NWO-VIDI Grant 917.36.351 to P.C.N.R., NWO Grant 908.02.097 to P.C.N.R., and Program Grant 903.39.291 to L.M.H.) and the Leiden Center for Cardiovascular Research (Gisela Thier Fellowship to P.C.N.R.).

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